"BABEŞ-BOLYAI" UNIVERSITY

CLUJ-NAPOCA

FACULTY OF PHYSICS

APPLICATIONS OF ELECTRON SPIN RESONANCE SPECTROSCOPY (ESR) IN THE STUDY OF SOME FOODS AND SOME COMPOUNDS OF PHARMACEUTICAL INTERETS

PhD Thesis Summary

Scientific Advisor:

Prof. dr. Grigore DAMIAN

PhD Student:

Ioan Csillag

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INTRODUCTION

Paramagnetism is a property of materials that have their own magnetic moments and which, without an external magnetic field, are randomly oriented and do not manifest at macroscopic level. But under the influence of an external magnetic field, these elementary moments are oriented towards the field direction and therefore their energy levels are splitting (the Zeeman effect).

This technique is able to provide details of molecular structure inaccessible to other analytical methods. This is due to the fact that the spin magnetic moment of the unpaired electron is very sensitive to local magnetic fields within the sample. The local magnetic fields usually come from nuclear magnetic moments of magnetic moments variations of some centers that may be present in the neighborhoods and can provide a number of interpretable information on the structure and the dynamics of an atomic or molecular system.

The possibility of detection of extremely small quantities of paramagnetic centers makes EPR technique to be extremely useful in the Chemistry study of the free radicals, paramagnetic ions in Physical Chemistry, Biochemistry and Biophysics various effects in Medicine and Physiology.

The present study attempted to extend the applications of ESR techniques and of specific methods to study the characteristics of some vegetable foods (strawberries, tomatoes and peppers) and of some compounds of phamaceutically interest, based on three research goals that can be addressed by this spectroscopic method;

- The detection and characterization of paramagnetic centers of microelements that are present in vegetable foods and pharmaceutical extracts;

- The characterization of antioxidant activities correlated with their ability to reduce the free radicals;
- The study of the effects of gamma radiation on some products such as food or pharmaceutical compounds.

The paper is structured in four chapters, in an attempt to address some applications of ESR in these research directions.In the 1st Chapter there are presented fundamental elements of the ESR spectroscopy based on the physical foundations of electron resonance phenomenon and on its theoretical interpretation. There are also presented some elements of spectroscopic technique in the detection and characterization studies of paramagnetic systems. There are highlighted the main technical and methodological elements specific to the application of ESR spectroscopy in the study of free radicals in the biopharmaceutical systems.

The 2nd Chapter of the paper is devoted to the presentation and characterization of the free radicals and antioxidants and of specific techniques for their study, by Electronic Spin Resonance usingthe spin traps and the nitroxide radicals. Free radicals can be detected directly, due to their low ability of recombination, and regarding the free radicals in the gaseous or liquid systems, the detection is done using indirect methods, due to their very short life time(microseconds). The hyperfine splitting of the ESR spectrum of the spin adduct provides qualitative and quantitative information regarding the original captured radical. By using nitroxide radicals and other stable radicals, it is possible to analyze various systems, providing valuable information in the study of the well-ordered systems, molecular dynamics study in amorphous systems and biological systems, the interface phenomena study in colloid systems or antioxidant character analysis of vegetable foods, pharmaceutical extracts, vitamins, minerals and other photochemical compounds.

In the 3rd Chapter, the thesis presents the experimental results of the application of ESR spectroscopy in the characterization of some vegetable

foods grown in the organic environment and in the greenhouse. Strawberries fruit are analyzed, tomatoes and peppers. The main aspects of the ESR spectra of the freeze-dried samples were highlighted and there were identified elements that may be fingerprints of the two ways of cultivation, namely the changes in the main components of the paramagnetic centers, such as iron, manganese and native-semiquinone free radicals. Also there are presented evaluations of the antioxidant activity of fresh juices of these vegetable food samples.

The 4th Chapter presents the results of applying the ESR spectroscopy in the study of some biomedical compounds. This chapter has two components, namely (i) the study of the antioxidant activity of the extracts of three herbs known to have therapeutic potential, namely Hyssopus officinalis, Ocimum basilicum and Teucrium chamaedrys and (ii) the study of the effects of gamma radiation on Purinetol,Atenolol and Pindololdrugs, in order to determine and quantify the radiation-induced effects in their structure.

In the end of the thesis there are presented the Conclusions of the experimental studies included in this paper, regarding some applications of ESR spectroscopy in the analyze of the samples.

1. PRINCIPLES OF THE ELECTRON SPIN RESONANCE SPECTROSCOPY (ESR)

1.1. Overview of ESR spectroscopy

ESR technique is based on induced transitions between Zeeman levels of paramagnetic system located in a static magnetic field. The splitter and the nature of the induced transitions are dependent upon the structure of the paramagnetic system and the external magnetic field value.

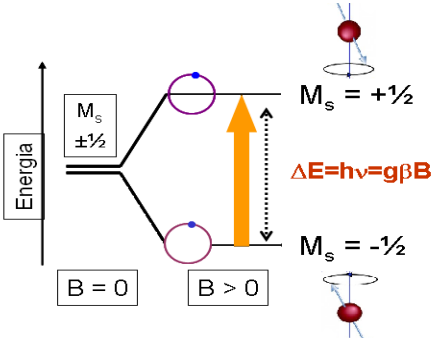


Fig. 1.1. Schematic representation of energy levels splitting in a magnetic field.

1.2. Spin Hamiltonian

ESR spectra can reflect different magnetic interactions, but all they actually originate from interactions between pairs of magnets of three types: electron spin, nuclear spin and magnetic laboratory. Thus, in the molecule, the electron, characterized by a magnetic moment, is not isolated, it is in an environment containing magnetic charges and moments that produce electric and magnetic fields around it. The electron interaction with these fields is described by the effective spin Hamiltonian.

1.2.1. Zeeman interaction

RES method is to study induced transitions between two neighboring Zeeman sublevels, subjected to a microwave field with a certain frequency called the resonance frequency [8]. ESR spectroscopy energy difference is due

to interaction of unpaired electrons with the magnetic field samples, effect called the Zeeman effect.

The absorption spectrum measured at the resonance condition is schematically represented in the lower part of the figure.

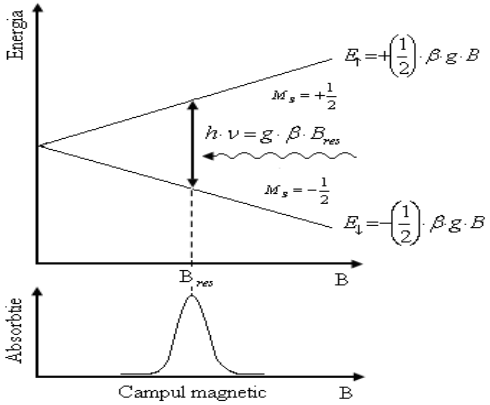


Fig. 1.2.1. Energetic levels and ESR resonance absorption for Zeeman interaction for one electron (S = 1/2)

1.2.2. The Electronic Zeeman interaction

The easiest form of the spin Hamiltonian is for a single electron with g izotrop. The Hamiltonian is called electronic Zeeman Hamiltonian, the term describing the electronic spin interaction with the applied magnetic field. In real chemical systems, however, the electron is associated with at least one atom, thus the Hamiltonian will have a more complex shape.

1.2.3. The Nuclear ZeemanInteraction

As electronic spin angular momentum interacts with the applied magnetic field, the I nuclear spin angular momentum will interact with the magnetic field applied [11], bringing the spin Hamiltonian at the form:

$$\hat{H}Zn = -g_N \cdot \mu_N \cdot B_0 \cdot \hat{I} \qquad (1.2.3.1.)$$

in which g_N and μ_N represent the nuclear g factor, respectively the Bohr nuclear magneton. The g_N factor may have both positive (proton) and negative values, as in the case of ^{15}N. Bohr nuclear magneton can be neglected in the ESR spectroscopy due to the fact that it has a much lower value than the electronic Bohr magneton.

1.2.4. Hyperfine interaction

The interaction between the spin magnetic moment and the nuclear magnetic moment of a neighboring nucleus is called hyperfine interaction [12, 13].

The isotropic part of the hyperfine interaction can be extracted directly from the EPR spectra in the liquid phase, providing information on the distribution of spin density of the electronic spin. The anisotropic part contains information about the distance between the electronic and nuclear spins, as well as their orientation to the applied magnetic field. This interaction may be sometimes mediated within liquids, while in the solid samples is determined by the analysis of the experimental spectra. As well asG factor, in solutions, the A factor provides information related to polarity and proticity of theneighboring microenvironment.

1.2.5. Superhyperfine splitting

Superhyperfine or hyperfine interaction of the ligand is due to the interaction between the spin magnetic moment and the nuclear magnetic moment of a neighboring nucleus (I_i) or the influence of several sets of equivalent nuclei (2nIi + 1).

As a result of this interaction thereare occurring additional splitting in the ESR spectra that are useful in obtaining information on covalent molecular system.

1.2.6. Spin-spin interaction of electrons

Considering a system with two or more electrons, to the spin Hamiltonian is added a term that characterizes the spin-spin electronic interactions. The interactions between different spin systems can be of two types:

- Dipole-dipole interactions, which are significant only at large distances, being relatively weak interactions, characterized by dipolar coupling tensor D

- Exchange interactions, occurring at small distances, of the order of interatomic connections, being more intense with several orders of magnitude than the dipole ones and they are characterized by exchange coupling tensor J

Dipolar interaction causes additional splitting even in the absence of an external magnetic field, also knownas zero field interaction, or fineinteraction.

1.3. The phenomena of saturation and relaxation

At thermal equilibrium, the probability of inducing a transition is proportional to the population of the spin states (Boltzmann distribution).

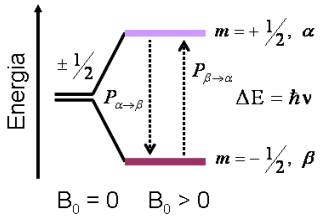
The populations of the two energy levels are almost equal, but with a slight excess of the lower level which leads to the energy absorption. To maintain an excess of population in the lower level, the electrons of the upper level lose an hν energy quanta in order to return to the lower level (Maxwell-Boltzmann law is satisfied in this way). By applying a microwave radiation an energy absorption occurs and the transition probabilities for absorption and stimulated emission are equal, then the populations will change their equilibrium values.

ESR spectra lines form provides a range of information regarding the speed of the analyzed processes. Thus, the spectral line width is due to the decrease in magnetization vector component in perpendicular plane. These energy dissipation mechanisms reflect the fact that nuclear spins are not in

thermal equilibrium with the environment and the return to equilibrum state occurs through a process of relaxation within a certain timeframe.

1.3.1. The phenomenon of saturation

The phenomena of saturation occurs when the excitation of the spin states by microwave power is faster than the speed of relaxation.



At thermal equilibrium, the transition probabilities for absorption and stimulated emission (P) are equal (coef. Einstein).

The amplitude of the function signal of the microwave power will depend onthe type of the relaxation process, as shown in Figure 1.3.1.1.

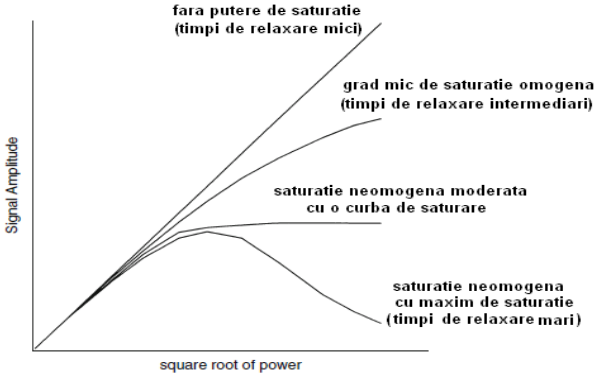


Fig. 1.3.1.1. RES signal amplitude dependence on the nature of relaxation processes

The determination of the saturation effect by increasing the microwave power, provides information on the relaxation properties of the system. If the microwave magnetic field has a sufficiently large amplitude and

is at resonance frequency, the two energy levels have approximately equal populations and the microwave absorption drops to almost zero, the so-called saturation condition being fulfilled (Figure 1.3.1.2).

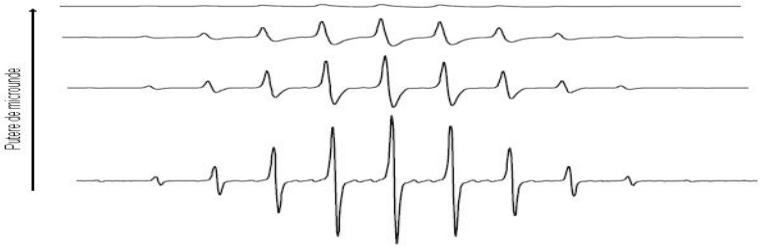


Fig. 1.3.1.2. ESR signal amplitude in the saturation process

1.3.2. Spin - latticeRelaxation

Spin - lattice relaxation is caused by fluctuations in the local magnetic field due to the motion of molecules. In this process the excess of energy is transmitted to neighbouring molecules as heat, due to molecular motion in liquids and gases and vibrations of atoms in solid networks. These fluctuations can drive change of states populating towards equilibrium, so the magnetization of the plane perpendicular returns to M_0equilibrium value, after a T_1time, called spin-lattice relaxation time. This process involves interaction between the spin system and the surrounding molecules (the energy is dissipated as vibrational energy network, rotation or translation) and is characterized by a T1relaxation time

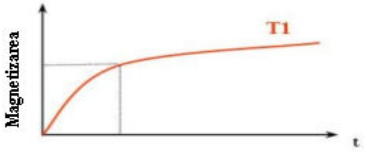


Fig. 1.3.2.1 Spin-lattice relaxation curve

As shown in Fig. 1.3.2.1, a high value of leisure time leads to a broadening of the absorption bands, while one that is too short leads to decrease in intensity of the absorption bands.

1.3.3. Spin - spin Relaxation

The spin-spin relaxation is caused by the spins distribution in the perpendicular plane, if they process at different speeds. Due to reduced magnetization within T_2time, the spectral line widens, width at medium height will be: $\Delta\nu = 1 / T2$ (process speed measurement) as shown in Figure 1.3.3.1.

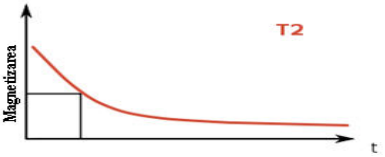


Fig. 1.3.3.1 Spin-spin relaxation curve

Spin-spin relaxation (or transversal relaxation) consists of dividing energy between neighboring nuclei as a result of spin-spin interaction and is characteristic to solids, being almost neglectable in liquids due to thermal motion of the molecules.

1.4. ESRExperimental technique

A general representation of the main components of an ESR spectrometer is shown in Figure 1.4.1.

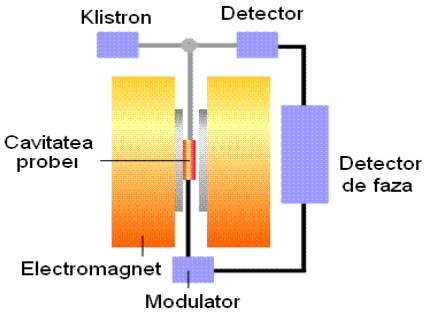


Fig. 1.4.1. The general scheme of ESR spectrometer

The functioning of the spectrometer is based on recording, in a static magnetic field,the induced polarization of an electromagnetic sample in microwave field due to quantum transitions between Zeeman levels of

paramagnetic particles. Thus, an ESR spectrometer consists of an electromagnet able to create a static magnetic field B, a microwave generator, a detector, a microwave cavity and a computer system for recording and observing the resonance phenomenon. All these elements are coupled through the sample to be studied, which is under the action of the static magnetic field and the microwave field.

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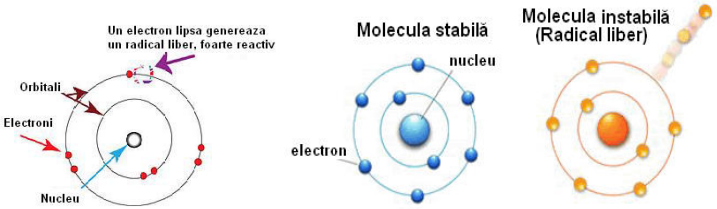
2. STUDY METHODS RES FREE RADICALS AND ANTIOXIDANTS

2.1. Free radicals

Radicals (also referred to as free radicals) are chemical species (atom, molecule or ion) which have an unpaired electron in the outer layer of the electronic coating, thus being strong chemical reagents that determine the instability of the atom / molecule containing them.



The free radicals occur in certain biological or chemical systems, in oxidation-reduction reactions in which occur major structural changes that lead to the change of the biological function of the respective substance (becomes more hydro soluble or intervenes in another chain of metabolic reactions).

2.1.1. Classification and general characterization of free radicals

The classification of the radicals can be done by various criteria: by lifetime and nature of the item that contains the unpaired electron, by type of orbital symmetry of the radical or by their nature. Such a classification that includes the majority of the radicals is the following one [1]:

A. By lifetime:

- Stable radicals;
- Persistent radicals;
- Transient radicals.

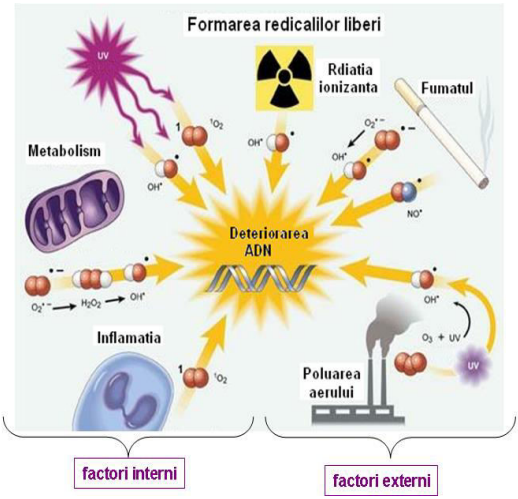
B. By the nature of the radical:

- Oxygen radicals;
- Nitrogen radicals;
- Aromatic compounds;

- Quinone and semi Quinone type compounds;
- Nucleic acids;
- Thiyl radical.

2.1.2 The generating of free radicals

The generating of free radicals in biological systems occurs as a result of the action of internal and external factors that the natural mechanisms of the biological entity cannot control. In fact, they are produced by the action of two sets of factors, namely internal factors and external factors. A schematic representation is shown in the below scheme [2]:



2.1.3. The main forming mechanisms of free radicals

There are a number of processes more or less complex which can generate free radicals. The free radical is easily formed when a covalent bond is broken and one electron remains with each newly formed atom, a process called bond cleavage. Depending on the type of covalent bonds (non-polar or polar) of the reactants and the nature of the substrate and the reaction conditions (polar or non-polar environment, gaseous or liquid state,

temperature, light) the bonds of the reactant can homolitical or heterolitical cleavage.

The main processes that generate radicals in organic and inorganic systems, may be summarized as:

• Homolysys;

• Photolysis and Radiolysis;

• Enzymatic reactions;

• Metabolism.

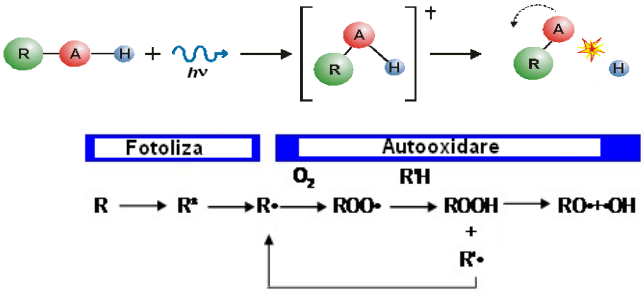
Homolysys

By homolitical cleavage of a covalent bonds, a molecule is divided into two, each fragment retaining one of the paired electrons.

Homolitical cleavage is uncommon in biological systems because it requires a large amount of energy, the source being either the ultraviolet light, heat or ionizing radiations.

Photolysis and radiolysis

The photolysis and radiolysis process is characterized by cleavage of one or more covalent bonds as a result of absorption of the electromagnetic radiation and the formation of reactive species (free radicals):



Enzymatic reactions

Enzymes are organic catalysts produced by living cells by acting on certain substances that are called substrates. The majority of enzymes catalyze the reaction of an organic substance with an inorganic free compound or yielded by other organic compound (water, phosphoric acid, hydrogen, oxygen etc.). Enzymatic reactions in the body release free intermediate radicalsthat react with each other or with other substances to form stable compounds.

Metabolism

Metabolism represents the totality of all biochemical and energy transformations that occur in the tissues of the living organisms. Free radicals occur in most cells of the body as a secondary product of metabolism, although some types of cells produce large amounts for specific purposes. The "foreign" chemical compounds of the body, called xenobiotics, are metabolized by xenobioticenzymes found in microsomes.

2.2. Antioxidants

Along with the increased interest in the study of free radicals, it has been developed a series of research regarding how to deal with their unwanted effects, in particular,the molecular oxidation. The oxidation reaction involves the transfer of electrons or protons from a substance to an oxidizing agent and, therefore, free radicals are formed. The reverse process is called reduction and,the result is a chemical process in which an atom accepts electrons.

The molecular compounds that inhibit the oxidation of molecules are called antioxidants and contribute decisively to reducing or combating the effects of free radicals. In biological systems, the main enzymes involved in neutralizing free radicals are superoxide dismutase (SOD), methionine - reductase and glutathione-reductase. [2].

The effects of antioxidants are known since long time ago; Mediterranean diet of fresh fruits and vegetables, good wines, fish and olive oil - mono - unsaturated has been associated with human health and welfare.

2.3. Specific methods of ESR spectroscopy in the study of free radicals and antioxidants

There are two ways of using ESR spectroscopy to detect free radicals, depending on their mobility and on the phase of the system that generates them, namely direct detection and indirect detection.

2.3.1 Spin markers method ("spin label")

An important group of molecules with outstanding magnetic characteristics in terms of both scientific and practical issue, is represented by stable nitroxide radicals or spin markers (spin labels). Synthesized for the first time about 40 years ago, nitroxide radicals currently play an essential role as spin probes in the study of biological systems, precursors in obtaining organic magnetic materials, control indicators in polymerization reactionsand in oximetry, contrast agents in tomography and even as therapeutical substances.

The nitroxide radicals are substances having a relatively high chemical reactivity, participating in a multitude of reactionsas substrate.

The fact that, in the case of nitroxide radicals, the unpaired electron is localized on the nitrogen nucleus (I = 1), in the magnetic field occurs a spectral structure made up of three lines (Fig. 2.3.1.2.).

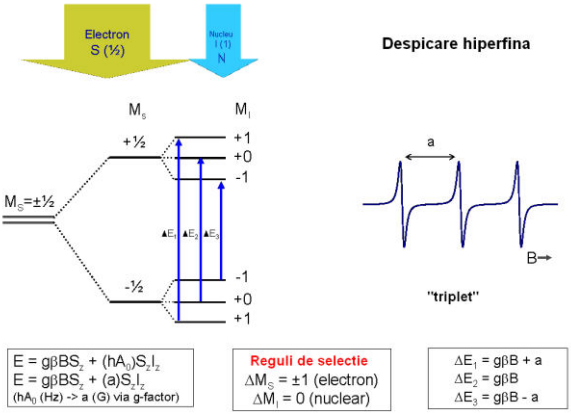


Fig. 2.3.1.2. Nitroxide radical hyperfine structure

The ESR spectrum analysis of the nitroxide radicals is related to three important aspectsof their utility, namely:

• study of the ordered systems where there are variations in magnetic parameters and spectral shape, which is directly linked to the characteristics of these systems,

• molecular dynamics study in amorphous systems or with varying degrees of heterogeneity (biological systems - the cell membrane, the protein systems etc.).

• analysis of the reduction velocity of nitroxide radicals and transformation in diamagnetic species (the study of antioxidant activity).

2.3.2. Spin trapping method ("spin traps")

Spin trapping method is a specific spectroscopic technique in which a free radical is trapped, by reacting with a double bond diamagnetic compound (Spin Trap). Thus, an intermediate nitroxide radical (spin adduct) is formed, which may be detected and characterized by ESR spectroscopy.

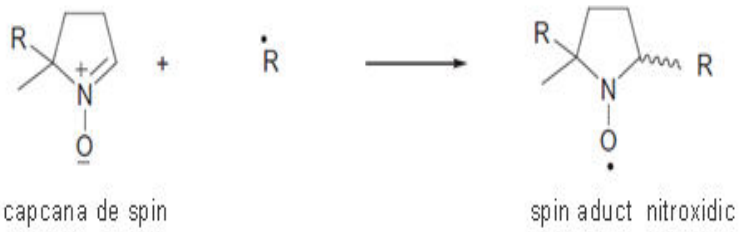


Fig. 2.3.2.1 Formation of the spin adducts

Capturing the spin is a kinetic experiment with ESR signal intensity reflected in the steady spin adduct state concentration at the time of spectrum registration. Therefore, the amount of spin trap required to obtain an optimal concentrationof the spin adduct will be closely linked by the formation and recombination rates of the free radicals in the studied systemreactions.

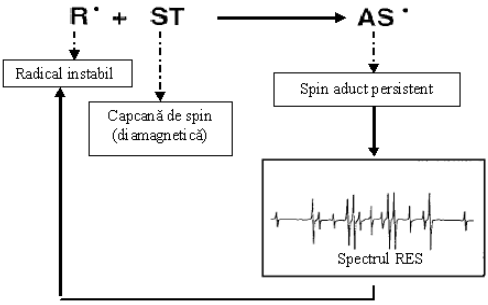


Fig. 2.3.2.2. Spin adduct formation

The spin trapping method may be used to determine the effectiveness of different anti-oxidants types compared to different types of free radicals, or for these free radicals detection in certain experimentalconditions.

For example, in Figure 2.3.2.3. is presentedthe spin adduct formation process and of the ESR spectrum for the DMPO/OH•system

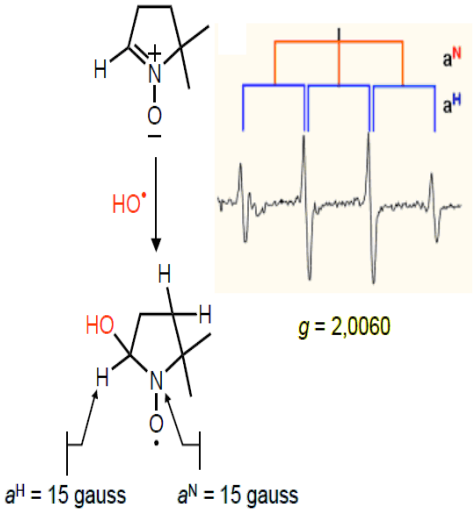


Fig. 2.3.2.3. Formation of the spin adduct and of the ESR spectrum for DMPO / OH • system

Nitrones and nitroso derivatives are usedas free radical collector agents (spin traps).

A schematic representation of the double integral of the ESR signal versus time is shown in Figure 2.3.2.4.

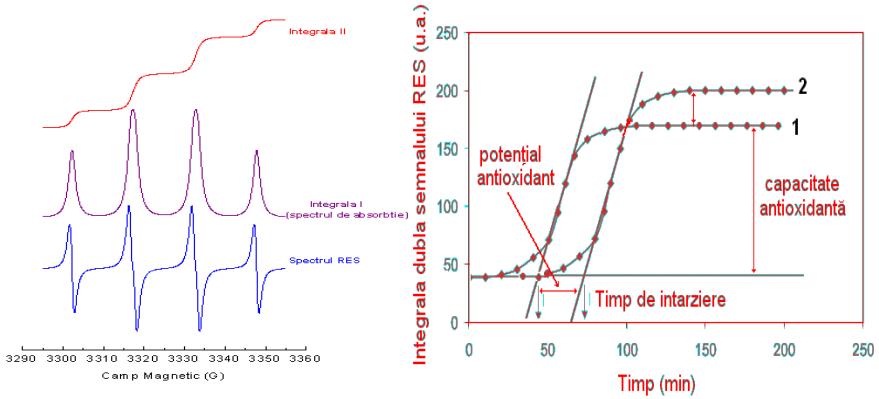


Fig. 2.3.2.4. Double integral representation scheme of ESR signal

The ESR technique of determining antioxidant activity consists in generating ●OH radicals by Fenton reaction (Fe^{2+}/ H_2O_2) and inserting this solution in the sample to studyin which was introduced a spin trap. By recording the EPR spectra from time to time it will be evaluated the delay time which is the period before the antioxidant activity of the extract is exhausted.

Thus, the antioxidant characteristics of the samples can be effectively monitored by removing the free radicals that were inserted in the study sample. This involves the characterization of the antioxidant activity kinetics.

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3. QUALITY ANALYSIS OF PLANT FOODS BY ESR SPECTROSCOPY

3.1. Food quality characteristics and methods of analysis

Overall the food quality is defined as an entity of great complexity, incorporating sensorial, physico-chemical, biochemical, microbiological and toxicologicalcharacteristics.

This is due to the fact that food, by its quality, has profound implications on life, representing an essential factor of metabolic processes and of the body'sbalance.

The spectroscopic methods (Atomic Absorption Spectroscopy (AA), Optical Emission Spectroscopy in inductively coupled plasma (ICP-OES), Emission Spectrometry in coupled plasma with mass spectrometry (ICP-MS), X-ray fluorescence, UV and Visible Spectroscopy (UV-Vis), IR Spectroscopy(MIR and NIR), Raman Spectroscopy, NMR, ESR Spectroscopy, Mass Spectrometry, Coupled Mass Spectrometry with gas chromatography (GC-MS) or liquid (LC-MS), Dichroism Circular (DC)) have become of great interest in evaluating the quality of agricultural products, especially food products [1]. These methods are very important for food component analysis because they require minimal amounts, do not require any special sample preparation and provide rapid analysis and also allow multiple tests on a single sample. Various research methods can highlight specific features and allow qualitative and quantitative evaluation of specific parameters [2, 3].

3.2. The study of some foods by Electron Spin Resonance Spectroscopy (ESR)

3.2.1. Basic and specific elements of ESR spectroscopy application

The Electron Spin Resonance (ESR), based on the absorption of electromagnetic radiation in the microwaves by paramagnetic molecular systems located in a homogeneous static magnetic field, allows the study of

paramagnetic systems and, therefore, may reveal some features of paramagnetic centers located in various matrix.

The study of paramagnetic centers of microelements

In the majority of the research,the study of paramagnetic centers of microelements from foods is made by liophylisation of the sample and direct recording of the RESspectrum, followed by the interpretations related to the type and characteristics of the sample.

In the case of vegetable products, the presence or the absence of persistent paramagnetic centers (paramagnetic ions of iron, manganese or semiquinone radicals) reflects a certain type of food cultivation. Thus, a solution for the spectral field assigned to manganese reflects an increased mobilityof the manganese ion (shortly bound in the protein environment) while a poor solving shows an interaction and stabilization in the protein centers [4].

The study of the antioxidant activity of the fresh or fermented foods or food extracts

The method used in research for assessing the antioxidant power of food products, is the persistent radicalsas nitroxide radical type and the DPPH (2,2 diphenyl- 1- picrylhydrazyl) as paramagnetic agents.

Nitroxide radicals are stable to oxidation, but they can easily be reduced to the corresponding hydroxylamines [5]. The standard redox potential of piperidine nitroxide derivatives (E0 = 0.2 eV) is high enough to oxidize biological compounds such as polyphenols, ascorbic acid, semiquinones and superoxide radicals.

This method consists in monitoring the reduction of the nitroxide radical by the antioxidant compounds of the studied samples. Thus, it appears that the number of paramagnetic species inserted in the sample disappears in time, withvarious decline rates and this is correlated with the content of the antioxidant compounds in the studied samples and obviously with the antioxidant characteristics.

Effects of sterilization of food products or of food spices by irradiation

The food irradiation is a food products treatamentof exposure to ionizing radiation to destroy harmful bacteria and other organisms and prolong their lifetime, having a benefic effect upon their quality and safety. The irradiation of different foods, including spices, with different doses of γ radiation has been used for many years in disinfection and microbial decontamination, in order to preserve food quality for a longer period.

Also, the electron spin resonance method (ESR) manages to highlight a number of private properties related to the electronic structure of the paramagnetic defect formed in irradiated solid network. Being a sensitive method for the detection of free radicals, it may also be used to study the mechanism of radiolysis or detection of drugs and irradiated foods [6, 7].

3.2.2. Characterization of some plant foods grown in organic environment and in the greenhouse, by ESR spectroscopy

3.2.2.1. Soil conditions in the cultivation of some food products

Scientific research on food quality and nutritive extracts are targets of major importance, both in terms of commercial trade and their impact on human health [8,9].

One of the most frequently asked questions about fruit and vegetable of the market is to distinguish those grown in greenhouses of the ones grown in the natural environment (organic) [10].

Most fruit and vegetables grown in greenhouses are grown in soil amended with compost and organic fertilizer. But these modified soils do not contain the full range of elements and essential substances [11].

3.2.2.2. Characterization of Strawberries fruit

The present study attempted to find specific items able to discern between two types of strawberries fruits, namely the ones cultivated in

greenhouse and the ones grown under natural conditions (organic), using ESR spectroscopy [4].

The ESR spectra of the liophylisated strawberries were recorded at room temperature and are shown in Figure 3.2.2.1.

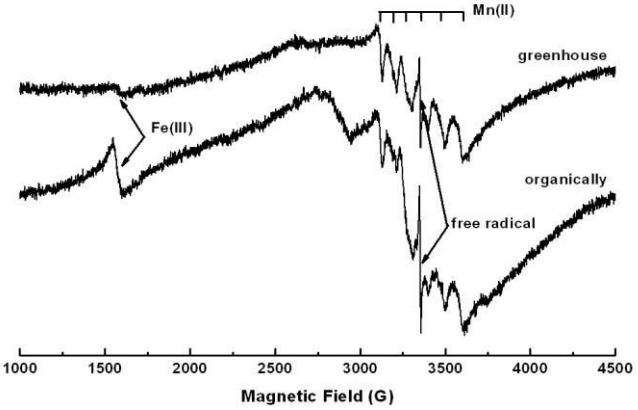

Fig. 3.2.2.1 ESR spectra of freeze-dried strawberry fruit

The main features of the ESR spectra are given by the presence of the characteristic signal for the free radical centered on the g = 2.004 and for paramagnetic centers of Mn^{2+} and Fe^{3+} with various signal intensities and precise degrees of solvingthe sextet lines forMn^{2+}.

Thus, if the strawberries grown in the greenhouse, feature Fe^{3+}signal is very weak, showing a small presence of iron in the paramagnetic state. This may be due to iron deficiency in the soil and environment (high pH, low organic matter content, soil low temperature) and to the fact that iron is absorbed by plant roots very low in the form of chelated iron (most fertilizers applied particularly in greenhouse crops are containing iron chelates) [12, 13].

The reaction rate of antioxidant compounds (fresh juice) and $ABTS^{+}$ was monitored by double normalized residual signal integrated ESR, which is

correlated with the number of paramagnetic species in time (fig.3.2.2.2).

The ESR spectra of the studied samples show differences in interpretation between the analized samples. Thus, we can say that, after analyzing the antioxidant activity of the studied extracts of strawberry, the antioxidant capacity of the strawberries grown in natural environment (organic) is significantly higher than those grown in the greenhouse. This result is emphasized by the kinetic reduction constant k ($k_{organic}$=0.09, $k_{greenhouse}$=0.058).

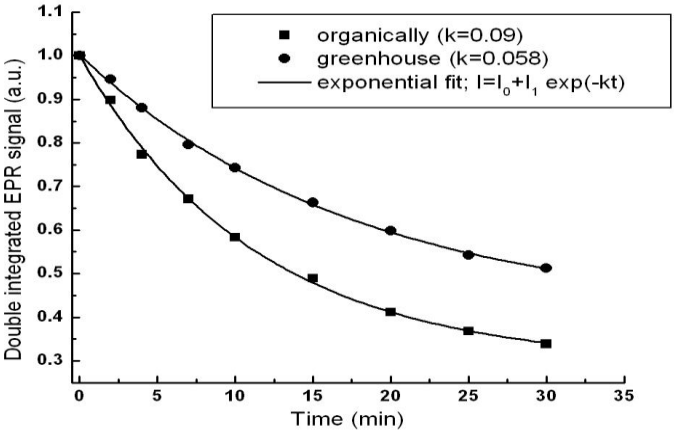

Fig. 3.2.2.2. Double integrated ESR signal variation in time (antioxidant activity of strawberries)

3.2.2.3. Characterization of tomatoes and peppers

The ESR spectra of the samples of tomato are shown in Figure 3.2.2.3.1.

The main characteristics of the ESR spectra are given by the presence of the signal characteristic for the free radical centered on g = 2.004 and of the paramagnetic centers of Mn^{2+} and Fe^{3+} with different signal intensities and particular solution levels for the sextet lines forMn^{2+}

For the tomatoes grown in organic environment it isalso observed an

overlap of a weak solved signal that can be assignedto some traces of copper ions.

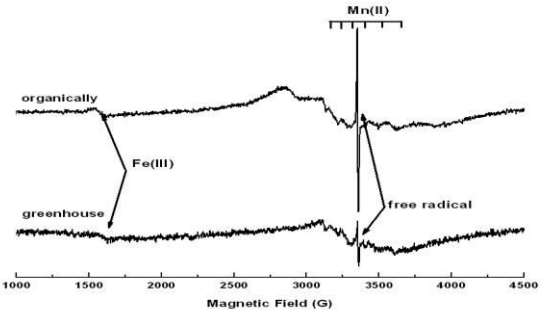

Fig. 3.2.2.3.1. ESR spectra of samples of freeze-dried tomatoes

As in the case of strawberry fruit, for the greenhouse grown tomatoes,the Fe^{3+}characteristic signal is very weak, showing a low presence of iron in paramagnetic state that can be correlated with the iron deficiency assimilated by the plant in the cultivation environment.

In the spectra of the pepper samples, there can be observed spectral characteristicshaving some interpretable differences. The spectra are shown in Figure 3.2.2.3.2.

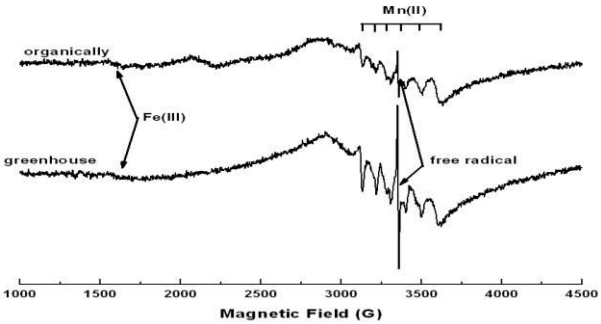

Fig. 3.2.2.3.2. ESR spectra of samples of freeze-dried bell peppers

The rate of reaction betweenthe antioxidants compounds of juice and $ABTS^+$ was monitored by following the ESR signal. To assess the antioxidant activity it has been usedthe double integral of ESR signal, which is correlated with the variation of the paramagnetic species
($ABTS^+$) in time. The result of these measurements is illustrated in Figures 3.2.2.3.3. and 3.2.2.3.4.

The graphical representationof the reduction kinetics was given by the reaction speed between the antioxidant compounds (fresh juice) and $ABTS^+$

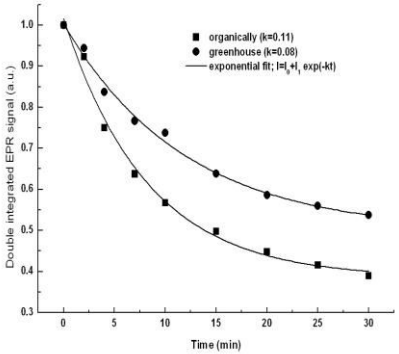

Fig. 3.2.2.3.3. Double integrated EPR signal variation in time (antioxidant activity of tomatoes)

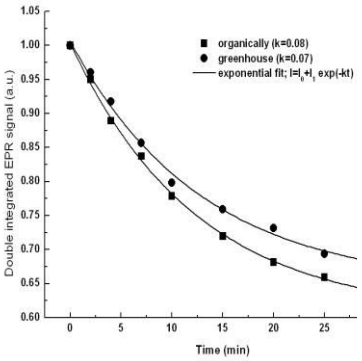

Fig. 3.2.2.3.4. Double integrated EPR signal variation in time (antioxidant activity peppers)

and was monitored by the double normalized residual integrated ESR signal, which is correlated with the number of paramagnetic species in time [14]. As in other situations the best result was obtained by fitting using exponential function of the first order.

The study of antioxidant activity of fresh juices show that the antioxidant capacity of food products grown in natural environment (ecological) is significantly higher than those grown in the greenhouse.

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4. THE ESR STUDY OF SOME PHARMACEUTICAL INTEREST COMPOUNDS

4.1. General aspects on the implementation of ESR spectroscopy in the study of pharmaceutical interest compounds

Under the name of biopharmaceutical compounds there are included those natural substances, plant extracts or synthesized substances, which demonstrate biological activity of organisms, only if the body already has these substances, in optimal doses. Some of these compounds are even produced in the body, while, on the other hand, the body can only benefit of others of the outside, the compounds getting into the body from food, from ingested fluids or oral administration.

4.2. The study of the antioxidant activity of some pharmaceutical plant extracts

Along with the research on free radicals there were also intensified the studies upon natural antioxidants due to their capacity to protect the organisms and the cells from damage caused by oxidative stress. In recent years researchers' attention was focused on the antioxidant products made from various herbs, spices [2-4] and other biological materials, which are of considerable interest because of their safety and of potential effects, both nutritive and therapeutic. In general, the antioxidants present in superior plants (vascular), are considered to be constituents having potential therapeutic value.

In the present paper were studied three herbal extracts known to have therapeutic potential, namely *Hyssopus officinalis, Ocimum basilicum* and *Teucrium chamaedrys*. The study of these extracts was complex and was done together with a team from the Faculty of Pharmacy of "Iuliu Haţieganu" University of Medicine and Pharmacy from Cluj-Napoca, targeting both their antimicrobial and antioxidant character of [5] .

Hyssop (Hyssopus officinalis) is a herbaceous plant of the family Lamiaceae, used both as a spice in culinary products, and in medicinal purposes. The plant is native to southern Europe, Middle East, reaching close to the Caspian Sea. The benefits of hyssop were known to old civilizations; even the contemporary name of the plant is adapted from a Greek term (hyssōpos = "holy herb"), which describes its use - hyssop leaves were used to clean the temples. As medicine herb, the hyssop herb is often used to treat bronchitis, asthma and has expectorant, antiseptic and healing properties.

Basil (Ocimum basilicum) is a plant native to tropical Asia, belonging to the Ocimumgenus, Lamiaceae family. The term comes from the Greek language, βασιλευς (basileus), meaning "king". It is said about this plant that it grew in the place where emperors Constantine and Helen discovered the Holy Cross. Therefore, the basil is also perceived as a sacred plant, both Orthodox and Catholic priests using it to be soaked in holy water and to wet things and people in order to give them blessings.

Wall germander (Teucrium chamaedrys) is a perennial plant that grows in meadows or forests regions in Europe, on the Mediterranean coast, both in the north and in the east. This plant has the appearance of a semi-shrub up to 40 cm, the leaves are green and have heavily toothed edges and the flowers are hermaphrodite, tubular, pink-violet. The wall germander is also known as 'jugărel', ‚dumbăţ' sau ‚sclipet', and belongs to the Lamiaceae family.

4.2.1. Sample preparation and determination of polyphenols content

The aerial parts of the plant at flowering time for Hyssop and Basil were collected from the experimental fields of the University of Agricultural Sciences and Veterinary Medicine from Cluj-Napoca and the Wall germander (Teucrium chamaedrys) plants were harvested in July 2013 from the spontaneous flora of Aries Valley.

An amount of 20 grams of each sample was used to obtain an extract, using 200 ml of 70% ethanol, for 30 minutes on a bath of water at 60°C. The samples were then cooled and centrifuged at 4500 rpm for 20 min. In order to analyze the content of polyphenols by other spectroscopic methods (Folin-Ciocâteu, UV-VIS and HPLC) stock solutions were prepared as reference standard of 10 mg of chlorogenic acid, p-coumaric, caffeic, cichoric, caftaric, ferulic, synaptic, gentisic gallic acid, rutin, quercetin, isoquercitrin, quercitrin, hyperoside, kaempferol, myricetol, fisetin, patuletin, apigenin, luteolin

dissolved in methanol and separated 10 ml flasks after a procedure described in detail in [5-7].

The total content of phenols (Total Phenolic Contents -TPC) of the extracts was measured using the Folin-Ciocalteu. Folin-Ciocalteu method is based on the oxidation of the studied extract using a molibdowolframat (Na_2WO_4 / Na_2MoO_4).

The total flavonoid content was determined and expressed using as standard the rutin (phenolic) and using the methodology described in the Romanian Pharmacopoeia [8].

The content of caffeic acid derivatives contained in the studied samples was determined by a spectrophotometric method using an Arnow regent (10 g of sodium nitrite, 10 g sodium molybdate in 100 ml of distilled water).

The results regarding the total content of polyphenols, flavonoids and caffeic acid derivatives grow in the following order

H. officinalis <O. Basilicum<T. chamaedrys

4.2.2. The analysis of the antioxidant characteristics

The antioxidant characteristics of the ethanol extracts of *H. officinalis, O. basilicum and T. chamaedrys* were studied using four techniques, namely spectrophotometrically method using DPPH, TEAC (Trolox Equivalent Antioxidant Capacity), the test HAPX (hemoglobin ascorbate peroxidase activity inhibition assay), and ESR (Electron Spin Resonance).

The recording of the spectra was done at room temperature. An illustrative representation of the decrease in the signals amplitude of TEMPO radical at various time intervals are shown in Figure 4.2.2.1.

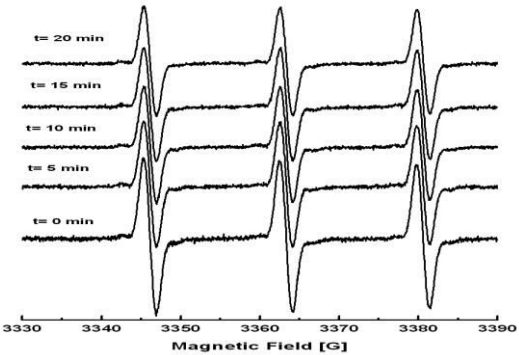


Fig. 4.2.2.1. TEMPO radical ESR spectra at various time intervals in sample H. Officinalis extract

Comparing the kinetic rates of the samples studied it can be seen that H. officinalis (HO) has the most significant antioxidant activity (kHO = 0.156) compared with the antioxidant activity of gallic acid (k_{AG} = 0.16), considered as standard and to which assessments can be made. Extracts of *O. basilicum* (OB) and *T. chamaedrys* (TC) had moderate antioxidant activity, kinetic constants having the values k_{OB}=0.068, respectively k_{TC}=0.049.

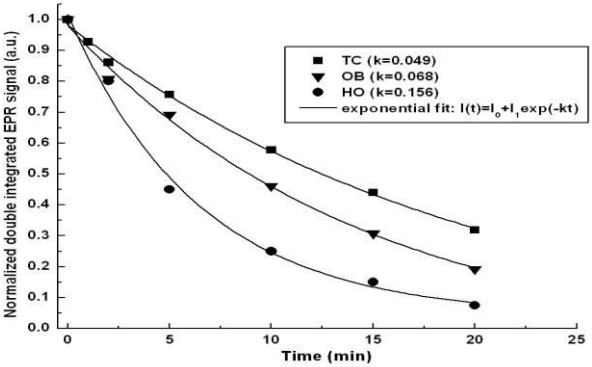


Fig. 4.2.2.2. Reprezentarea vitezei de scădere a intensităţii semnalelor RES a extractelor deTeucrium chamaedrys(TC), Ocimum basilicum(OB) şi Hyssopus officinalis(HO)

4.3. The study of the Gamma radiation effects upon some drugs

Purinethol [1,7-dihydro-6H-purine-6-thione, $C_5H_4N_4S \times H_2O$] or 6-mercaptopurine is an antineoplastic agent that inhibits the purine metabolism (inhibiting uric acid production). It is used in chemotherapy, for the treatment of acute leukemias, both in the induction of remissions and, in particular, in the maintenance therapy of acute lymphoblastic leukemia and acute myelogenous leukemia.

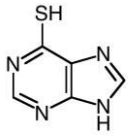


Fig.4.3.1. Chemical structure of Purinethol

The ESR spectra of the PURINETHOL powder irradiated with gamma radiation (fig.4.3.2.), demonstrate the presence of more stable paramagnetic species, their relative concentration depending on the absorbed dose [9].

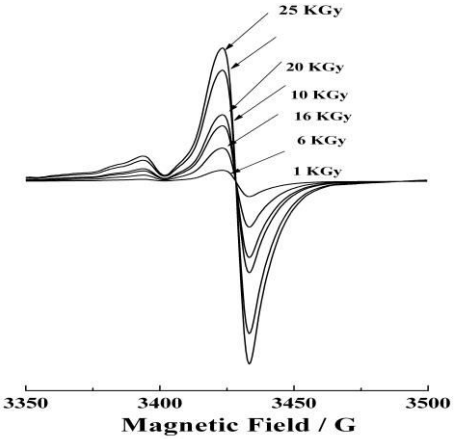


Fig. 4.3.2. EPR spectra of gamma irradiated at different dosages Purinetolui

To determine the specific magnetic parameters, the spectrum was simulated with the POWFIT program, starting from a minimum configuration of paramagnetic species (Fig. 4.3.3.).

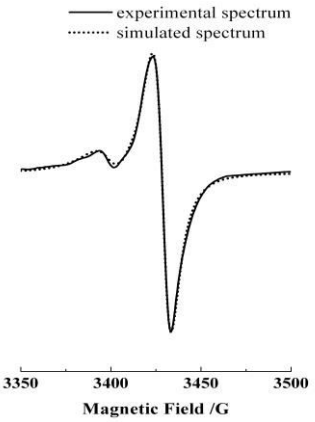


Fig. 4.3.3. EPR spectra of gamma irradiated to 20KGy Purinetolui, experimental and simulated

The spectrum mainly seems to belong to the RSO- sulfinyl radical radical formed by the oxidation of Thyl radical. The spectrum Anisotropy is probably due to the location of radicalic centers on both aromatic rings, causing a local axial arrangement. In addition, as a result of the simulation, it is observed that beside the Thyl radical, there are two more paramagnetic species. Both radical species appear to be oxygen-centered radicals, that interact with a β proton and a γproton.

The radical concentration increases as a result of a polynomial function of 4th order, so that, after a significant increase in absorbed doses of up to 15 kGy, there is a slower change at a high level of irradiation dose, up to reaching a plateau phase (Fig.4.3.4.) which corresponds to the saturation of all radical species. It seems that during irradiation the radicals are recombining within rapid processes, other than those which occur in the absence of radiation (according to radiolysis). The relative integral of intensities of the ESR absorption spectra was obtained by fitting the parameters, and was given by the following relative relation:

$$I(D) = I_0 + I_1 D + I_2 D^2 + I_3 D^3 + I_4 D^4$$

where I represents the corresponding value for the concentration of free radicals to a certain amount, k is the kinetics rate of reaction and D is the absorbed dose.

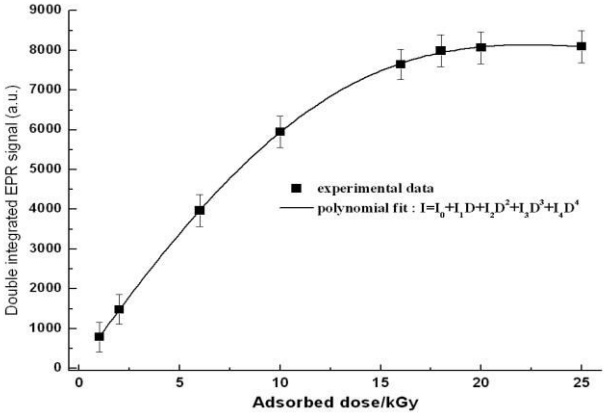


Fig. 4.3.4. Curve dose-response of gamma irradiated Purinetol

Beta-blockersdrugs

Atenolol (other names: Ablok, Atenalon, Atenolol, Atenopress, Ateneo, Plenacor) is a hydrophilic substance. These is less digestive absorbed (50%) and is low biotransformed at firstmetabolism pass (~ 0%).

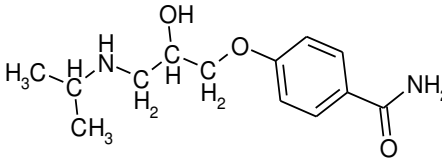


Fig. 4.3.5. Chemical structure of Atenolol

The Atenolol has low absorbsotion rate by food and antacids with aluminum. Its elimination is via the kidney, with a half-life $T_{1/2}$= 9 hours. It inhibits with particular selectivity the $beta_1$ receptors (compared with the $beta_2$receptors). The therapeutic dosages, especially the small ones, selectively act upon the heart, which is an advantage when arrhythmias coincides with asthma [10].

Pindolol (other names: Durapindol, Pectobloc, Pindololum, Visken) is a non-selective beta-adrenolytic (depending on the affinity for adrenergic receptors), having a clear sympathomimetic effect. It has an oral absorption and bioavailability of more than 90%. It is unchanged after first-pass metabolism. It is eliminated by the liver (60%) and kidneys (40%), $T_{1/2}$= 3 - 4 hours. Its action is prolonged for elderly people and in hepatic impairment [10].

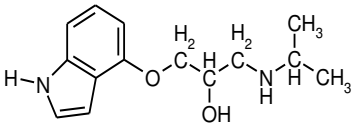


Fig. 4.3.6. Chemical structure of Pindolol

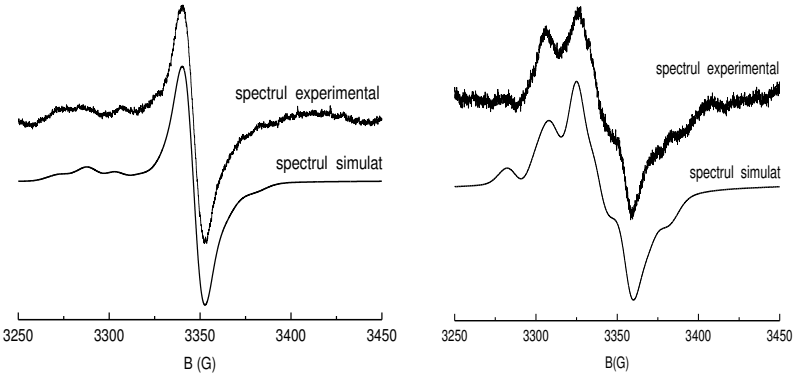
By γiradiation were generated free radicals which were characterized by ESR spectroscopy [1]. For their analysis there were made simulations of the experimental spectra based on a minimum configuration of the number of radical species. In Figure 4.3.7. there are presented the experimental and simulated spectra of the beta-blockersstudiedsamples.

The ESR spectrum obtained for the Pindolol sample represents a sum of corresponding spectra to all the free radicals that are simultaneously present in the sample, being dominated by a large central line. The broad signal observed is characteristic forthe free radicals trapped in a solid matrix [11].

The ESR spectrum obtained for the Atenolol sample has been assigned to the overlap of two radicals spectra. The first radical consists of a triplet centered at g = 2.003 ± 0.0005 with a line width ΔB = 9.8 G, because of two protons equivalent to the hyperfine splitting constant a_1 (H) = a_2 (H) = 16.3 G. This radical was assumed to be in good agreement with the isotropic coupling, generally found to carbon-centeredπ radicals, having $R-\dot{C}H_2$ form and being produced by the hydrogen moving from the group (methyl-).

For the second radical, the unpaired electron can be localized on the nitrogen atom of the Imidazole group, leading to a characteristic of the hyperfine splitting a_N = 16 G – 18 G and g = 2.009 ± 0.0005

Therefore, even if the studied drugs are part of the same therapeutical family and have quite similar chemical structures, following the irradiation, they do not present the same types of free radicals. This was also noticed by other researchers, for other types of drugs of beta-blockers class.



(a) *(b)*

Fig. 4.3.7. ESR spectra of gamma irradiated (a)Pindolol, (b) Atenolol

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CONCLUSIONS

Using Electron Spin Resonance spectroscopy (ESR) it was possible to study the nature and characteristics of the paramagnetic centers of microelements that are present in some foods of plant origin, through sample lyophilization and direct recording of ESR spectrum, followed by interpretations related to the type and characteristics of the sample. The study of strawberries, tomatoes and peppers showed that:

• the presence or absence of persistent paramagnetic centers (paramagnetic ions of iron, manganese or semiquinone radicals) reflects a certain type of cultivation of the food.

• the shape and degree of resolution of ESR spectral structure gives information on metabolic assimilation modality that can be correlated with specific ways of cultivation (in the open environment, greenhouse, nutrient management mode etc.). This way, a resolution of the spectral domain assigned to manganese reflects an increased mobility of the manganese ion (very little bound in the protein) while a poor solving shows an interaction and a stabilization in protein centers.

• the detection and characterization of paramagnetic species can be an important indicator (a spectroscopic fingerprint) in detecting fruit grown in the greenhouse and those grown under natural conditions.

The proposed method for qualitative analysis of antioxidant activity is based on the monitoring on reduction in time, the persistent radicals Tempo (*(2,2,6,6-tetrarnethyl-l-piperidinyloxy*), $ABTS^{+}$cationic radical (*2,2'-Azinobis (3-etilbenzotiazolin- 6-sulfonic acid*)) and DPPH *(2,2- diphenyl- 1-picrylhydrazyl*). The decrease of the relative concentration of paramagnetic species (stable radical reduction) is obtained by double integrating the experimental spectra. The number of paramagnetic species shows a decrease in time with different rates, this fact being correlated with the antioxidant activity of the studied samples. For the studied samples, juices or ethanol extracts from

some pharmaceutical interest plants, the reduction kineticsof the stable radical is given by a first order kinetics in which the specific parameter ofeach type of extract and type of processing is the k reaction kinetics parameter, representing the speed of oxid-reduction of radicals in time. This parameter is a measure of the antioxidant characteristics of the sample studied. However, for a more complete characterization of the antioxidant activity and more accurate understanding of the chemical and physical factors that play a significant role in the antioxidant activity of the studied samples is necessary to use additional techniques (method of Folin-Ciocalteau, HPLC etc.). Following the research within this thesis there have been revealed the following results:

• The study of antioxidant activity of fresh juices showed that the antioxidant capacity of naturally grown food (organic) is significantly higher than those grown in the greenhouse.

• For ethanol plant extracts, it can be observed that H. officinalis (HO) has the most significant antioxidant activity (kHO = 0.156) compared with the antioxidant activity of gallic acid (k_{AG} = 0.16), considered as standard and to which assessments can be made. Extracts of *O. basilicum* (OB) and *T. chamaedrys* (TC) had moderate antioxidant activity, kinetic constants having the values k_{OB}=0.068, respectively k_{TC}=0.049.

A important application of the ESR spectroscopy approached in this thesis is to study the effects of gamma radiation on some of medicines, namely Purinetol, Atenolol and Pindolol, in order to determine and quantify the radiation-induced effects in their structure. The research provides useful information in the technological processes of manufacture, sterilization and storage of medicines, medical management methodology and terms of validity. There can be obtained important data regarding the influence of preparation technology upon the generating of free radicals, of pharmaceutical forms uponthe time stability and of the factors influencing this stability. The results can be summary as follows:

• the most important reaction that contributes to Purinethol degradation involves the oxidation of sulfur atom at position 6 of the purine ring. Beside thyl radical, there are two more paramagnetic species, having magnetic parameters that appear to be specific to oxygen-centered radicals, which interact with a β proton and a γproton.

• beta-blocker drugs (Atenolol and Pindolol), although part of the same therapeutical family and have pretty similar chemical structures, following irradiation they do not present the same types of free radicals. For Pindolol compound, the value of the g isotropic factor, obtained - g_0 = 2.005±0.0005, is characteristic to radicals centered on (carbon-) or (nitrogen-). The ESR spectrum obtained for the Atenolol sample has been assigned to the overlap of two radicals spectra. The first radical consisting of a triplet, was assumed to be in good agreement with the coupling isotropy, typical for the carbon-centered π radicals,having the form $R-\underset{\bullet}{C}H_2$ form and being produced by the hydrogen moving from the group (methyl-). The second radical, has the unpaired electron localized on the nitrogen atom of the Imidazole group, leading to a characteristic of the hyperfine splitting (a_N = 16 G – 18 G).

Contributions to the thesis

Articles indexed in the ISI

1. Laurian Vlase, Daniela Benedec, Daniela Hanganu,Grigore Damian, **Ioan Csillag**, Bogdan Sevastre, Augustin C. Mot,Radu Silaghi-Dumitrescu and Ioan Tilea **(2014)**, *Evaluation of Antioxidant and Antimicrobial Activities and Phenolic Profile for Hyssopus officinalis, Ocimum basilicum and Teucrium chamaedrys*, *Molecules*, 19(5), 5490-5507; doi:10.3390/molecules19055490, (FI=2.416)
2. Laura Bolojan, **Ioan Csillag**, Vasile Miclaus, Grigore Damian, *Free radicals investigation in γ-irradiated Purinethol (6-MP)*, *Farmacia*, acceptată spre publicare (IF=1.251)
3. **Ioan Csillag,** Grigore Damian**,** *EPR study of the strawberries grown in the greenhouse and organically*, Studia UBB, Seria Chemia (trimisă spre publicare) (IF=0.191)

Volume of scientific conferences

1. G. Damian, V. Miclăuş, Laura Bolojan, **I. Csillag**,*Detection an characterization of free radicals in some gamma irradiated drugs and foods by EPR spectroscopy*, *Proceedings of the10th International Symposium on Metal Elements In Environment, Medicine and Biology , November 11-12, 2010,* Publishing House "Eurobit" Timisoara, **pp.115-120**, ISSN 1583 – 4204

Participation in scientific conferences

1. G. Damian, V. Miclăuş, Laura Bolojan, **I. Csillag**, *Free Radicals Investigation in γ-Irradiated Purinethol,* „The X-th National Conference of Biophysics (CNB 2009)" Cluj-Napoca, 1-3 0ctober 2009.
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4. **Ioan Csillag,** Grigore Damian**,** *EPR study of the strawberries grown in the greenhouse and organically*, Advanced Spectroscopies on Biomedical and Nanostructured Systems, Cluj-Napoca, September 2013

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ESR spectroscopy, free radicals, antioxidants, strawberries, peppers, hyssop, basil, wall germander, purinethol, atenolol, pindolol.